Exploring body fluid biomarkers for Diagnosis, Prognosis and Treatment Monitoring in MS

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General objective

In MS, there are only very few body fluid biomarkers that are accepted as reliable and practicable parameters so far. Biomarkers play a role in determining MS (e.g. cerebrospinal fluid-specific oligoclonal bands (OCB)), in differential diagnosis (e.g. anti-aquaporine-4 and anti-MOG serology in NMO-SD), in monitoring disease modifying treatment (DMT) (e.g. anti-interferon antibodies and anti-natalizumab antibodies) and predicting adverse effects (screening anti-JCV antibodies in natalizumab treatment for the risk of PML).

The problem of diagnosing MS is largely solved by using MRI, clinical presentation and neurological assessment which led to the formulation of the revised diagnostic criteria.[1]

However, making a timely diagnosis can still be complicated in a minority of patients. This may occasionally result in an undesirable late diagnosis and subsequently delayed treatment. To date, validated and discriminative body fluid biomarkers reflecting and predicting natural disease progression and disease activity are lacking.[2, 3] Such biomarkers are crucial for personalized medicine and may provide guidance in the rapidly evolving treatment armamentarium in MS.

The main aim of this thesis was to explore novel biomarkers that are associated with disease progression and to explore biomarkers in monitoring treatment and treatment safety. With this aim different markers reflecting potential roles in neuroinflammation, demyelination, and axonal damage were analyzed. For monitoring DMT and DMT safety natalizumab concentrations and neurofilament light were explored.

During this search, two biomarkers with a more diagnostic purpose were also worthy to analyze namely kappa free light chains and mRNA-sequencing of blood platelets. After all, biomarkers useful in determining MS can likewise be potential biomarkers in predicting disease progression and treatment monitoring.

The following section summarizes the main results of our biomarker studies in MS. In addition, main findings are discussed in relation to the existing literature, and clinical implications and future perspectives will be addressed.

PART 1 Diagnostic biomarkers

Chapter 1.
Kappa free light chains, a valid tool in the diagnostics of MS.

In the latest 2017 revisions of the McDonalds criteria the OCB have a prominent role in patients with clinically isolated syndrome (CIS). However, the assessment of OCB is labor intensive, requires trained personnel and is in some cases examiner- and method-dependent, which may affect its reliability. The question raised: 'Is there a biomarker that can be measured with an automated and quantifiable procedure?'

Since the late 1970s, multiple studies have reported increased CSF levels of kappa free light chains (KFLC) in MS[4-14] and due to the development of the more sensitive nephelometric and turbidimetric FLCs assays[4-8] KFLC and lambda free light chains (LFLC) can be easily detected. However, until our study described in **chapter 1**, a large multicenter study to validate this biomarker was lacking.

In **chapter 1** we validated KFLC and LFLC indices as a diagnostic biomarker in MS compared with OCB in a large multicenter study including samples from eighteen MS-centers across Europe (219 controls and 526 CIS/MS patients) with a known OCB status. We measured KFLC and LFLC in paired CSF and serum samples. We defined cut-offs for abnormal FLC indices and based on the defined cut-off, subjects were classified as positive or negative for kappa or lambda FLC as binary result. The sensitivity, specificity and accuracy between the two different diagnostic tools (OCB and FLC) were compared.

We found a cut-off for the KFLC index of 6.6, and for the LFLC index a cut-off of 6.9. Compared to OCB the KFLC-index is more sensitive (.88 vs .82) at the cost of a lower specificity (.83 vs .92). This resulted in a higher negative predictive value for the KFLC-index compared to OCB, but a lower positive predicted value. This suggest that the KFLC-index is a valid test for diagnosing MS. In addition, our results indicated that the LFLC-index is not a valid test for diagnosing MS.

Discussion and future perspectives

Validating FLCs in a large multicenter study is necessary assuming it can be a valid tool as a potential cost-effective replacement of the OCB. The sensitivity and specificity found in **chapter 1** for the KFLC index in CIS/MS were lower compared to other smaller studies.[13-17] However, in these previous studies, there was a less heterogeneous control group (except in the study described by Senel et al. 2019). For example, similar as for the OCBs, KFLCs can be elevated in inflammatory controls[18], and thus specificity will be lower when included. The

large number of patients and the large heterogeneous control group in our study gave us a reflection of the real-life clinical situation thus avoiding spectrum bias and allowed us to give a more representative sensitivity and specificity for OCB, KFLC and LFLC indices.

It is challenging to establish cut –off values for biomarkers. The cut-off in this study was calculated using a data-driven Gaussian mixture modeling approach. This is a different approach compared to other studies. We reasoned that the cut-off should be defined by biological levels and not based on clinical diagnosis, since the latter contains clinical uncertainty due to lack of a golden standard. The cut-off found in **chapter 1** for KFLC is in line with results from previous multi-center studies showing a KFLC-index cut-off of 5.9 and 7.0.[8, 13] This almost comparable cut-off for the KFLC index in two multi-center studies supports its robustness and implies that it may be used as an universal cut-off.

The last two decades multiple revisions were published of the McDonald MS criteria. This resulted in that not all included patients were diagnosed based on the same MS criteria in our study, which may have influenced the diagnosis of CIS patients particularly. We addressed this problem by pooling all CIS and MS patients. We performed several sensitivity analyses in CIS or MS patients separately, and by reclassifying and excluding specific clinical groups. No relevant differences were seen when comparing MS to control group.

We concluded that for clinical practice, the KFLC index is more accurate in excluding CIS/MS compared to OCB but for ruling in a diagnosis of CIS/MS, analysis of OCB appears to be more accurate. By replacing OCB with KFLC in diagnostic practice there is a slightly higher chance that a patient with a diagnosis different from MS will get the diagnosis of MS and maybe unnecessarily exposed to potential negative side effects of early treatment. Since the KFLC-index is more sensitive at the cost of a lower specificity, we should stress that replacement of OCB by the KFLC-index is not optimal to arrive at high diagnostic certainty.

An advantage of the higher sensitivity of KFLC, is that an earlier diagnosis and subsequent treatment start may be considered. It may be an option to start treatment based on the KFLC result and clinical/MRI findings according to the novel McDonald criteria, or to use KFLC results in patients on treatment when a switch may be considered while on DMT. This could be considered as a subject for future studies.

One important note is that the best set up for a future study would be to include a test population of suspected MS cases instead of already diagnosed cases. The use of the KFLC values as a predictor for CS conversion to MS is demonstrated in several studies.[19-21] However, a recent study showed that FLC concentrations at CIS diagnosis were not significantly higher in CIS-converters.[13]

Since 1995 different papers have been published exploring KFLC values as a disease progression marker. Different studies found that KFCL predicted subsequent physical deterioration in MS patients.[22-24] Nonetheless, one study did not find evidence for a relation of KFCL with disease progression.[25] The role of KFLC in predicting disease activity or disease progression is interesting for future studies using large cohorts of patients with long-term follow up.

Chapter 2.
mRNA-sequencing of blood platelets as novel diagnostic biomarker in MS.

So far, no blood biomarker has been convincingly confirmed as a useful tool in the diagnostic work-up of MS. Blood-based approaches harbor obvious advantages and recently, the long-neglected blood platelets, being the second most abundant cell type in peripheral blood, have shown emerging potential as a new source for biomarker discovery in several diseases, but not yet for MS.[26, 27]

During the final stages of platelet production, platelets are loaded with pre-messengerRNAs before developing from the megakaryocyte.[28, 29] As a result, platelets contain a rich messengerRNA (mRNA) repertoire that can change during megakaryocyte development but also during platelet formation and platelet circulation. Especially the change of RNA transcripts during circulation, possibly achieved by specific splicing queues, is of relevance in this chapter.

Platelets are known to respond to activating signals from their environment with specific splicing of their pre-mRNAs and uptake of RNA from different cell types, leading to a unique and dynamic RNA repertoire [30-35]. The diagnostic potential of this specific splicing of mRNAs in platelets has already been demonstrated, as recent findings have shown that mRNA sequencing from tumor educated blood platelets distinguishes healthy controls (HC) from cancer patients with an accuracy of 95% [36].

Platelets seem to not just be involved in inflammatory and immune responses but may also contribute to the pathogenesis of MS.[37-39] As a result of their involvement in immune response and apparent causal role in the progression and development of the disease, we hypothesized in **chapter 2** that blood platelets contain a disease specific mRNA signature, thereby investigated their potential as diagnostic biomarker for MS. In this chapter, we isolated and sequenced platelet RNA of blood samples from 57 MS patients and 66 age- and gender-matched healthy controls (HCs). 60% of the matched samples were employed to develop a particle swarm-optimized (PSO) support vector machine classification algorithm.

The remaining 40% of the samples served as an independent validation series. In total, 1249 RNAs with differential spliced junction expression levels were identified between platelets of MS patients as compared to HCs. The spliced platelet RNA was subsequently used as input for the development of a diagnostic MS classifier capable of detecting MS with >80% accuracy in the independent validation series (n=50, AUC: 0.87, 95%-CI: 0.77-0.97, p<0.001).

Discussion and future perspectives

The blood-based approach presented in this chapter could assist in the diagnosis of MS without being as invasive as CSF collection, therefore allowing for easy disease determination in case of a diagnostic challenge. Ultimately, it would be favorable if blood platelets mRNA could replace CSF OCB to substitute the requirement of fulfilling dissemination in time on MRI according the 2017 revisions of the McDonald criteria.[1]

To our knowledge, our study was the first utilizing mRNA found in circulating platelets as a blood-based biomarker for distinguishing MS patients from asymptomatic individuals. Recently, the protocol determining mRNA in platelets was published, enabling the MS community to test platelet RNA for diagnostic algorithm development.[40]

Obviously, there are limitations that need to be discussed concerning our data. First, aside use of age- and gender-matched asymptomatic controls, no individuals were included with other auto-immune or (neuro-) inflammatory disease potentially reducing the diagnostic accuracy. Second, all MS patients have been diagnosed with the disease for a minimum of ten years. Third, the sample size analyzed is still relatively small, potentially resulting in algorithm overfitting. To reach true clinical relevance, additional studies should also focus on early-stage MS cases and CIS patients to assess the early-detection potential. Despite above mentioned problems, we provided evidence for the clinical potential of circulating blood platelet derived mRNA as liquid biomarker for RRMS. More studies are needed, however, to assess the performance of this diagnostic tool in various presentations of MS. Also, platelet derived mRNA may have potential as progression marker in MS and as a response marker in DMT users. We are currently expanding our measurements to our treatment cohorts.

PART 2 Disease course biomarkers

Chapter 3.
Exploring cerebrospinal fluid mtDNA concentration as a biomarker in MS disease progression and activity.

In this part of the thesis we explored markers reflecting disease course, with a focus on disease progression.

In recent years, impaired mitochondrial function is increasingly recognized as a key pathological hallmark of MS.[41, 42] Demyelination leads to an increase in energy demand in order to maintain an appropriate intra-axonal ion balance and could thereby affect the number, transport and activity of mitochondria.[43-46] The number of mitochondria is highly increased in chronically demyelinated axons as well as in reactive astrocytes[43, 45] and extensive neuronal mtDNA deletions have been observed in MS cortical brain samples.[47] Based on the observations that mitochondrial dysfunction plays a crucial role in MS pathology and the possible role of mitochondrial dysfunction in clinical disease progression, we explored in **chapter 3** the potential of mtDNA levels in the CSF as a candidate biomarker of identifying patients with progressive disease in a Dutch cohort (50 RRMS patients, 40 PMS patients, 23 non-inflammatory and 7 inflammatory controls).

The main conclusion of this chapter was that concentrations of free circulating mtDNA copies are increased in CSF of patients with progressive MS compared with non-inflammatory control patients. Also, there was a trend ($p=0.08$) for a modest positive correlation with Expanded Disability Status Scale (EDSS) specifically in progressive MS patients. In addition, we showed that patients with a high T2 lesion volume displayed higher mtDNA concentrations compared to patients with a relative low T2 lesion volume. The group with lower normalized brain volumes showed higher mtDNA concentrations compared to patients with higher normalized brain volumes, suggesting a positive correlation between the concentration of free circulating mtDNA copies and brain atrophy. Altogether, our data may suggest that increased concentrations of cell free mtDNA are associated with MS disease activity and progressive disease.

Furthermore, we explored the effect of disease modifying treatment on free mtDNA levels in longitudinally obtained CSF samples in a Swedish cohort (42 RRMS patients, 20 other neurological disease controls and HC). We showed that patients treated with fingolimod had significantly decreasing (almost 50%) mtDNA copy levels at follow-up compared to baseline.

Discussion and future perspectives

Previous studies showed reduced levels of mtDNA in both Alzheimer[48, 49] and Parkinson[50] disease cases and it has been speculated that a decrease in mtDNA might be a common phenomenon observed in neurodegenerative diseases. In contrast to this, elevated mtDNA levels as we found in PMS, have also been detected in CSF samples from children with traumatic brain injury and were highly predictive of a poor outcome.[51] This might suggest that high values of free circulating mtDNA in CSF can be seen as a potential biomarker of acute cellular and mitochondrial stress. It is nowadays widely accepted that neurodegeneration and concomitant brain atrophy are common pathological features of MS, particularly in the progressive phase of the disease. Demyelination leads to an increase in axonal energy demand, which may superimpose effects of neurodegeneration in MS, which is a possible explanation for the higher concentrations of mtDNA in progressive MS in this chapter. However, a recent study analyzing mtDNA in PMS, showed that when mtDNA was analyzed in ventricular CSF (post mortem) at the end-stage of progressive multiple sclerosis mtDNA levels were depressed.[52] This study used a small sample size and comparing post mortem with samples taken from living patients warrants cautious interpretation of the data.

The cellular origin of enhanced mtDNA levels in the CSF of progressive MS patients found in our study is yet unknown, however it is conceivable that mtDNA is released upon neuro-axonal injury or oligodendrocyte damage, as these are prominent features of progressive MS. Alternatively, mtDNA might be secreted into the extracellular compartment by extracellular vesicles derived from distinct CNS cells, such as reactive astrocytes, particularly in lesions that are packed with mitochondria.

In line with our results, Varhaug et al. showed increased levels of cell free mtDNA in CSF of MS patients compared with controls.[53] Further, they found an inverse correlation between the duration of the specific symptoms and levels of mtDNA, concluding that increased mtDNA concentration may reflect early, active inflammatory activity. Increased mtDNA levels in the CSF might also contribute to the disease process by activating an immune response. Mitochondrial DNA is a damage-associated molecular pattern (DAMP), which can bind to glial Toll-like receptor-9 and trigger an inflammatory response.[54] Hence, it is conceivable that enhanced mtDNA levels in the CSF might elicit a glial immune response. Fissolo et al. further explored the potential role as a diagnostic and disease activity biomarker in MS by measuring CSF cell-free mtDNA levels in a large cohort of individuals with relapse-onset and progressive clinical forms of MS, patients with CIS and control subjects.[55] No significant differences were observed between MS patients, CIS patients and neurologic disease controls. Within the CIS group, mtDNA levels did not significantly differ between CIS patients who converted to MS and those who continued as CIS. Similar to our results, no significant differences were observed between relapsing and progressive forms of MS. Within the MS group, they showed that mtDNA levels were similar between patients in relapse and

remission, patients with and without gadolinium enhancing lesions, and patients with and without progression on the EDSS score during follow-up.

All the above mentioned results signify that additional research is needed to explore the functional effects and use of mtDNA concentrations in the CSF of MS patients.

A possible role of mtDNA as a biomarker of fingolimod treatment response can also be the subject of future studies. It is likely that fingolimod reduces inflammation-mediated cellular damage and subsequent release of mtDNA based on the significant decrease of mtDNA after initiating fingolimod. However the precise mechanism underlying reduced mtDNA CSF levels upon fingolimod treatment warrants further investigations and CSF as source for monitoring treatment effects has its obvious drawbacks. Also, further comparisons with other disease modulatory treatments are needed in order to understand if this effect is specific for fingolimod or a generic response to reduced inflammation and concomitant CNS cell injury.

Chapter 4.
Acid sphingomyelinase, no potential marker for disease progression.

Recent evidence suggests that alterations in the sphingolipid pathway may reflect disease activity in MS.[56, 57] Due to the activity of enzymes essential in the sphingolipid pathway, such as sphingomyelinases, ceramides of different chain lengths may be produced and participate in different cellular processes such as differentiation, proliferation and programmed cell death.[58, 59] Increased ceramide levels have been detected in CSF of patients with MS.[60, 61]

An altered protein expression of acid sphingomyelinase (ASM) in MS brain tissue samples (obtained at rapid autopsy and immediately frozen in liquid nitrogen or fixed in formalin) has been demonstrated in a previous study. They identified that reactive astrocytes are the primary source of enzyme activity and subsequent ceramide production.[57]

The study in **chapter 4** explored the potential of acid sphingomyelinase (ASM) activity levels in the serum as a candidate biomarker to identify MS patients with an active or progressive disease course. Furthermore, we explored several targets of the sphingolipid metabolism in relation to DMT.

Levels of serum ASM activity were longitudinally analyzed in 40 CIS, 64 RRMS and 10 primary progressive MS patients (PPMS), and 22 HC. ASM activity and sphingolipid levels were measured in a different sample of 61 RRMS patients using DMT. Analyses of ASM activity

levels showed that when pooling all types of MS, a significant higher ASM activity level was observed than in HC. The levels did not significantly differ in the serum between patients with RRMS, SPMS and PPMS and we did not find an association between ASM activity and the annualized relapse rate, disease activity, MRI variables or EDSS progression.

In the second part of this chapter we investigated the association between the sphingolipid metabolism and DMT. Despites ASM activity levels did not reflect treatment response, we did observe a significant increase of two types of ceramides (Cer-$C_{16:0}$ and Cer-$C_{24:0}$) and four types of sphingomyelin (SM-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$) during fingolimod use.

Discussion and future perspectives

The activity of ASM allows conversion of sphingomyelin into ceramides. Ceramides may induce neuronal mitochondrial dysfunction and axonal damage by participating in different cellular signaling cascades and processes such as differentiation, proliferation and programmed cell death.[58, 59] So far, ASM activity levels have only been determined in the CSF of MS patients, demonstrating increased levels compared to other neurological diseases (OND).[62] Besides higher levels of ASM activity, the number of exosomes that carry ASM in the CSF was significantly higher in MS patients than in patients with OND and this was correlated to CSF ASM activity.[62]

In contrast with other studies[60, 62], we did not observe correlations between EDSS and ASM or other components in the sphingolipid metabolism. In these previous studies the markers were measured in CSF, suggesting more potential for CSF than serum with respect to suitable MS biomarkers.

Regarding the possible effect of fingolimod on reducing the production of pro-inflammatory lipids (such as ceramide), we could not confirm this within **chapter 3**. Only one previous study explored the effect of fingolimod (and other MS therapies) on different sphingolipids in MS patients[63]. They observed that IFN-ß treatment strongly increased plasma levels of Cer-$C_{16:0}$, Cer-$C_{18:0}$, Cer-$C_{20:0}$, and Cer-$C_{24:1}$ compared to healthy controls, untreated patients, or patients receiving fingolimod or natalizumab medication.[64]

One hypothesis why increasing levels of ceramides and sphingomyelin were observed, in 25 treated MS patients in our study, is that fingolimod is an S1P mimicking agent and thereby may decrease levels of endogenous S1P, which in turn may lead to increasing levels of ceramides and sphingomyelins due to the sphingolipid rheostat.

Although higher levels in MS patients were found, we concluded that ASM activity levels did not show potential as a biomarker for predicting disease activity, progression or response

to DMT. Two ceramides and four types of sphingomyelin require further investigation as potential markers for treatment response.

Chapter 5.
Serum tissue transglutaminase associates with disease progression.

Tissue Transglutaminase (TG2) is a Ca2+-dependent crosslinking enzyme, regulated by inflammatory mediators.[65-67] Recently, the presence of TG2 in infiltrating MHC-II positive cells in MS lesions was demonstrated [68] as well as increased TG2 mRNA in MS patient-derived monocytes[69] suggesting a possible role for TG2 in the pathophysiology of MS. The clinical implications of TG2 have already been described for several human diseases. [70-72] Nevertheless, the clinical implication of TG2 in MS patients has not been studied yet. The aim of **chapter 5** was to assess whether TG2 expressed by peripheral blood mononuclear cells (PBMC) is altered in patients with MS and whether this correlates with measures of disease activity and progression.

In this chapter, peripheral blood mononuclear cells (PBMCs) were isolated from 151 HC and 103 RRMS patients, 36 secondary-progressive MS patients (SPMS) and 22 PPMS patients. We observed that TG2 mRNA levels were differentially expressed in healthy controls compared to RRMS patients and that the mRNA levels were associated with disease progression measured as either high EDSS score (std ß=0.26; *p*=0.02), normalized brain volume (NBV, std ß=-0.18; *p*=0.02), normalized white (NWMV, std ß=-0.17; *p*=0.03) and grey matter volume (NGMV, std ß=-0.15; *p*=0.03) in MS patients. In addition, in PPMS patients, TG2 mRNA levels were also associated with T1-hypointense lesion volume and T2-lesion volume. Our results suggest that PBMCs-derived TG2 mRNA levels can be used as a biomarker for multiple sclerosis progression, especially for PPMS.

Discussion and future perspectives

We observed no differences in TG2 mRNA levels between MS patients and HC subjects. Nevertheless, TG2 showed significant associations with EDSS as well as NBV, NGMV and NWMV at baseline thus suggesting that TG2 mRNA levels could represent a quantitative measure of neuronal loss. No significant evidences were found for a contribution of TG2 in disease activity measured as both relapse or annualized relapse rate (ARR) in RRMS patients. Those data suggest that alterations in TG2 mRNA levels are not attributed to the inflammatory phase of the disease. In addition, a trend toward expressing lower TG2 mRNA levels was observed in RR patients with active disease (presence of gadolinium enhancing lesions at the baseline) who represent the most inflammatory group of MS patients. In line with those findings, TG2 mRNA levels were not affected by the applied DMT, which target

infiltration of immune cells in the CNS and/or the production of inflammatory cytokines. Those results were unexpected as TG2 expression is known to be modulated by inflammation and inflammatory mediators.[65-67, 73] An association profile similar to what was observed in the whole MS patients group was also observed in PPMS patients, where TG2 correlates also with T1-hypointense lesion volume and T2-lesion volume. Those data could suggest that in particularly in PPMS patients, alteration in PBMC-derived TG2 mRNA expression are associated with progression of the disease and ongoing axonal damage.

To characterize TG2 expression in PPMS patients, the expression profile of full-length TG2 and TG2 splice variants in PBMCs of PPMS patients were compared to those of HC subjects in an additional study[74] carried out by our own researchers at the MS center Amsterdam. The TG2 variant V4b was significantly higher expressed, and both V4a and V4b variants were relatively more expressed in relation to full-length TG2. These observations open new avenues to unravel the importance of TG2 alternative splicing in the pathophysiology of PPMS.

Furthermore, when pooling RRMS with SPMS patients we observed that TG2 mRNA was significantly associated with clinical disability (EDSS) and MRI measurements (normalized brain volume and normalized white matter volume). Interestingly, in this pooled group, TG2 association with NGMV was not significant. Remarkably, in both RRMS and RRMS/SPMS patients, TG2 mRNA levels showed predictive power for the course of the disease. In fact, TG2 mRNA was associated with worsening of the clinical symptoms (change of EDSS) over a 2 year follow-up. Future studies in an independent cohort are needed to validate our findings. In addition, a prospective study with repeated and long-term measurement of PBMC-derived TG2 mRNA would help to evaluated the stability of TG2 mRNA in time and the robustness of the assay.

PART 3 Treatment monitoring biomarkers

Chapter 6.
Serum neurofilament light in natalizumab treated MS patients.

As already mentioned, biomarkers reflecting and predicting disease activity and progression are crucial for personalized medicine and may provide guidance in the rapidly evolving treatment armamentarium in MS.

Natalizumab (NTZ)(Tysabri, Biogen Inc, Cambridge, MA, USA) is a humanized monoclonal antibody constraining the migration of leukocytes over the blood–brain barrier and it is

well known the be very effective in MS treatment.[75] Several studies showed a decrease in the annualized relapse rate (ARR) and stabilization[75-77] or even improvement in physical disability. [78, 79] In contrast, a recent study showed that when taking into account early inflammation and the impact of natalizumab on disease activity during the initial treatment phase, a higher than expected proportion of patients treated with NTZ showed disability progression.[80]

Neuroaxonal injury may be found in several neurological disorders and is accompanied by release of neuron-specific neurofilament (NF) proteins into extracellular space. These proteins can leak into CSF and into blood and can reach abnormal levels as a result of axonal damage in neurodegenerative, inflammatory, vascular and traumatic diseases.[81, 82]

Neurofilament light (NfL) has increases in CSF and serum of RRMS patients during relapses, returning to baseline within a couple of months of the acute event.[83-86] There are findings that serum NfL (sNfL) levels are also associated with EDDS progression. [84, 87] It remains unclear, however, whether sNfL may, in addition, predict or reflect disability progression in the absence of relapse-related neuroaxonal damage. In **chapter 6** we investigated the potential of sNfL as a biomarker of disability progression in the almost complete absence of, or with limited contribution of acute focal inflammation in a cohort of 89 NTZ-treated RRMS patients. We examined whether sNfL at initiation and after 12 months of treatment predicted disability progression in the following 2 years and whether the longitudinal trajectories of sNfL levels differed in individuals with disability progression from individuals without. In this chapter we observed a significant reduction in sNfL levels at 3 months (almost 50%) and reached its nadir within 12 months after NTZ initiation. We found no difference in the longitudinal dynamics of sNfL levels in patients with or without progression based on EDSS or EDSSplus. sNfL levels at initiation and at 12 months did not predict EDSS or EDSSplus progression in the following 2 years.

Discussion and future perspectives

The significant reduction in sNfL that we observed in **chapter 6** after initiation of NTZ is similar to other studies[84, 87, 85] However, the focus on long-term disease progression in a population without remaining disease activity has been a rather new approach. Recently, in a cohort of MS patients, sNfL was reported to predict EDSS worsening in the following year, [87] regardless of DMT status at time of measurement. In our NTZ cohort, sNfL levels at BL, and at 12 months did not predict EDSS progression in the following 2 years and sNfL levels at BL and 12 months did not differ significantly between both groups. In the study by Barro et al[87], DMT status was heterogeneous at time of sNfL measurement, with some patients treated, and others not. In our cohort, all patients were untreated for at least 2 months prior to sNfL assessment at BL. Moreover, in the present study, all patients were treated during the assessment of progression, uncoupling damage arising from focal inflammatory activity

from neurodegeneration. Our results are supported by the other studies[88, 89] They showed that no significant correlations were measured concerning EDSS progression and baseline sNfL levels.

For future plans, atrophy measurements need to be taken into consideration as it seems that brain atrophy and sNfL levels are correlated.[88, 89] In our study, atrophy measurements were not available yet.

The strength of this study is the prospective nature of the detailed longitudinal clinical and radiological data and longitudinal sample collection. However, the study also has possible limitations. The data would have benefited from a larger sample size and longer duration of follow up. Nevertheless, the complete lack of a trend towards a difference in longitudinal sNfL measurements between progressors- and non-progressors strengthens the truthfulness of our findings. In conclusion, our findings confirm the potential of sNfL to monitor the reduction in focal inflammatory damage that accompanies NTZ introduction, but fails to capture longer-term EDSS worsening ("silent progression") that is largely independent of relapse activity.

Chapter 7.
Treatment response markers: serum natalizumab concentration and lymphocyte count during treatment in RRMS patients switching from natalizumab to fingolimod.

Natalizumab is an effective treatment in relapsing-remitting multiple sclerosis, but is associated with an increased risk to develop progressive multifocal leukoencephalopathy (PML). PML is a severe, potential lethal disease, caused by the John Cunningham (JC) virus. Mainly because of the risk of PML, a substantial proportion of JC virus positive patients switch to fingolimod. The main question in switching from natalizumab to fingolimod is, what the optimal wash-out (WO) period is between the two treatments. Previous reports show a clear benefit when the duration of a WO period of natalizumab is 0-3 months in comparison to longer WO periods. However, there is no consensus regarding the optimal duration of a WO period under 3 months. In **chapter 7** we compared MS disease activity after different WO periods. In addition, we investigated several factors that possibly influence recurrence of disease activity, including serum natalizumab concentration and lymphocyte counts.

From a prospective observational cohort study of natalizumab treated patients we selected 52 patients who switched to fingolimod. We divided the patients in three groups (<6 weeks n=16, 6-8 weeks n=18, >8 weeks n=18 WO). Serum natalizumab concentration and lymphocyte count were assessed during and after natalizumab treatment.

Patients with a WO period of >8 weeks had a significant higher recurrence of disease activity (OR 6.8, 95% CI 1.4–32.8) compared to patients with a WO period of <6 weeks. Serum natalizumab concentration and lymphocyte count did not predict recurrence of disease activity.

Discussion and future perspectives

The results we observed in **chapter 7** confirm earlier studies that report an increase of disease activity after 2-4 months of WO period in comparison to shorter WO periods.[90-95] Ideally, to limit the risk of recurrence of disease activity, the WO period should be short enough to allow fingolimod to be clinically effective before natalizumab concentration drops under therapeutic levels. The downside of such a short WO period is the ongoing risk of PML in JC virus positive patients after discontinuation of natalizumab and how fingolimod could possibly increase the risk of PML and negatively influence the course of this serious complication.

All current literature regarding WO periods when switching from natalizumab to fingolimod recommend a delay of less than three months because of the risk of recurrence of disease activity.[90-94, 96] We estimated that the possible increased risk of PML with a decrease of 1-2 months of WO period does not outweigh the significant reduction of disease recurrence. Therefore, we recommend a WO period of less than 6 weeks when switching from natalizumab to fingolimod. Extra precautions regarding the risk of PML in JC virus positive patients may be taken, such as a baseline MRI and repeated scans 3 and 6 months after cessation of natalizumab and timely testing of JC virus DNA in the cerebrospinal fluid in case of suspicious MR activity.[96]

We hypothesized that lower concentration of NTZ would predispose to disease recurrence after NTZ discontinuation. However, we found no significant difference in concentration under natalizumab treatment when comparing patients with disease activity and patients without disease activity, which is in agreement with a recently published study of 12 patients switching from natalizumab to fingolimod.[97] In our study, the possible explanation could be that, the clinical disease activity reappeared after three months of natalizumab discontinuation because the concentration of the drug had already decreased under therapeutic levels.[92] When comparing longitudinal natalizumab concentration in patients with and without disease activity, the mean concentration at three months was lower in patients who did not experience disease activity (0.8 versus 4.2 μg/ml).

When switching from natalizumab to fingolimod, we hypothesized that clinical effect of fingolimod might be delayed due to the relative lymphocytosis natalizumab causes in a proportion of patients.[98] However, this study showed no correlation between lymphocyte count and disease activity. T cell subsets are unknown in this study, so describing the

fingolimod-mediated changes on CD4+ and CD8+ T cells was not possible. The lack of correlation between an overall lymphocyte count and disease activity will not rule out the probability that the changes in subpopulations of lymphocytes (including CD4+ / CD8+ ratio) may have impact on the recurrence of disease activity.

To determine the optimal WO period in case of switching from natalizumab to fingolimod, larger randomized trials are needed, preferably comparing different WO periods, including one arm starting fingolimod immediately after the final natalizumab infusion. However, in future studies the influence of the WO period on the risk of PML will still be difficult to establish given the rarity of this serious complication. Future biomarker studies in switchers should include serum NfL as this biomarker may capture both the recurrence of MS disease activity as well as damage due to early (carry-over) PML.

Chapter 8.
Natalizumab concentrations

As mentioned in chapter 7, natalizumab is an effective treatment in relapsing-remitting multiple sclerosis. All patients receive the same treatment regimen of 300mg every four weeks, despite differences in pharmacokinetics between individual patients. In this treatment regimen, natalizumab concentrations may stay detectable in serum in up to 200 days after cessation of therapy.[99] Serum natalizumab concentration corresponds with the percentage of α-4 integrin receptor saturation.[100] Desaturation of the α-4 integrin receptor occurs when the serum NTZ concentration falls under 1-2 µg/ml.[100] Above this threshold of 2 µg/ml, natalizumab receptor saturation will roughly fall between 70-100%.[100] An adequate receptor saturation is estimated as ≥70-80% saturation, although prospective data confirming this assumption are lacking.[101, 102] Based on a model with results from a large phase II trial, approximately 90% of patients showed natalizumab trough concentrations largely exceeding 2.5 µg/ml. Levels exceeding 2.5 µg/ml could indicate that the approved treatment regimen of natalizumab for RRMS results in patients receiving more natalizumab than necessary for optimal drug efficacy.[103, 104] Furthermore, it is suggested that higher natalizumab receptor saturation could increase the risk of PML.[105] This unconfirmed hypothesis leads to clinicians extending dose intervals in natalizumab treatment with the aim to reduce the PML risk by decreasing natalizumab exposure.[105-107]

The aim of the study in **chapter 8** was to measure natalizumab serum concentrations and correlate concentrations with disease activity and possible influencing factors. We explored if natalizumab concentration has potential as a marker of treatment response.

In **chapter 8**, natalizumab serum concentrations were measured in serum of 80 patients from a prospective observational cohort study. Data on demographics, duration of treatment, EDSS, clinical exacerbations, brain MRI and body weight were collected.

We measured high (≥10µg/ml) natalizumab concentrations in 94% of patients. Intra-individual concentrations were stable. The spread in concentrations between patients was substantial and did not correlate with disease activity. We found a negative association between natalizumab concentration and body weight (β=-0.30, p=0.010).

Discussion and future perspectives

In part 3.3 of this thesis natalizumab concentrations were explored as potential treatment monitoring markers.

The variation in natalizumab concentrations was substantial. However it did not correlate with disease activity (**chapter 8**). The mean natalizumab serum concentration in our cohort was above 20 µg/ml which is in agreement with recently presented data.[108] The mean concentration was the same in patients with active versus non-active disease, which suggests that high concentrations do not result in an increase of treatment efficacy in comparison to lower but still adequate concentrations. Considering this and the large proportion of high natalizumab concentrations, natalizumab could perhaps be administered less frequently (or with a lower dose) to reach natalizumab concentrations that are lower but still cause adequate receptor saturation and consequently, optimal drug efficacy.[100] Caution is advised though, because of a large spread in concentrations and the well-established rebound effect which occurs after cessation of natalizumab treatment.[109] Therefore, monitoring natalizumab serum concentrations during treatment should be essential.

Extended dose intervals could help reduce costs of medication and increase quality of life for the patient with fewer hospital visits, but further studies are needed to establish the safety of alternative treatment regimens. At this moment a prospective multicenter single-arm trial with one-year follow-up and an extension phase of one year (ClinicalTrials.gov; NCT03516526) is completed at the MS Center Amsterdam (Amsterdam University Medical Center, Vrije Universiteit), with the aim to evaluate if natalizumab efficacy is maintained when switching to personalized extended interval dosing based on individual natalizumab trough concentrations in stable RRMS patients (reference: Neurology in press).

Results of such trials will hopefully give a decisive answer to the question if extending dose intervals in natalizumab treatment is feasible without losing drug efficacy.